



**EFFECT OF INOCULUM DENSITY AND INDUCTION SUBSTRATE
CONCENTRATION ON CELLULASE PRODUCTION BY CO-CULTURES OF
Trichoderma reesei QM9414 AND *Aspergillus phoenicis* QM329**

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ABSTRACT: The cellulase enzyme complex of hyperproducing strains of *T. reesei* is deficient in β -glucosidase, which can result in accumulation of inhibitory levels of cellobiose. Supplemental β -glucosidase from *A. phoenicis* grown in co-culture with *T. reesei* has been used to overcome this problem. However, the competitive interactions between the two fungi has not been addressed adequately. A factorial experiment was conducted to evaluate the effects of inoculum density (4 levels of actively growing mycelia) and induction substrate concentration (0, 0.25, 0.5, and 1% starch) of *A. phoenicis* on the hydrolytic potential of the extracellular cellulase enzyme complex from 7 day old co-cultures upon alkaline peroxide pretreated wheat straw. Production of β -glucosidase was greatest for co-cultures with the highest inoculum densities and starch concentration. The cellulolytic potential was greatest for high inoculum densities at lower starch concentrations. Co-cultures with highest inoculum at 0.25% starch gave the highest cellulose conversion efficiency (69%). Our data suggest that these results are due to the overproduction of *A. phoenicis* biomass at the expense of *T. reesei* during the growth phase in cultures containing high starch levels.

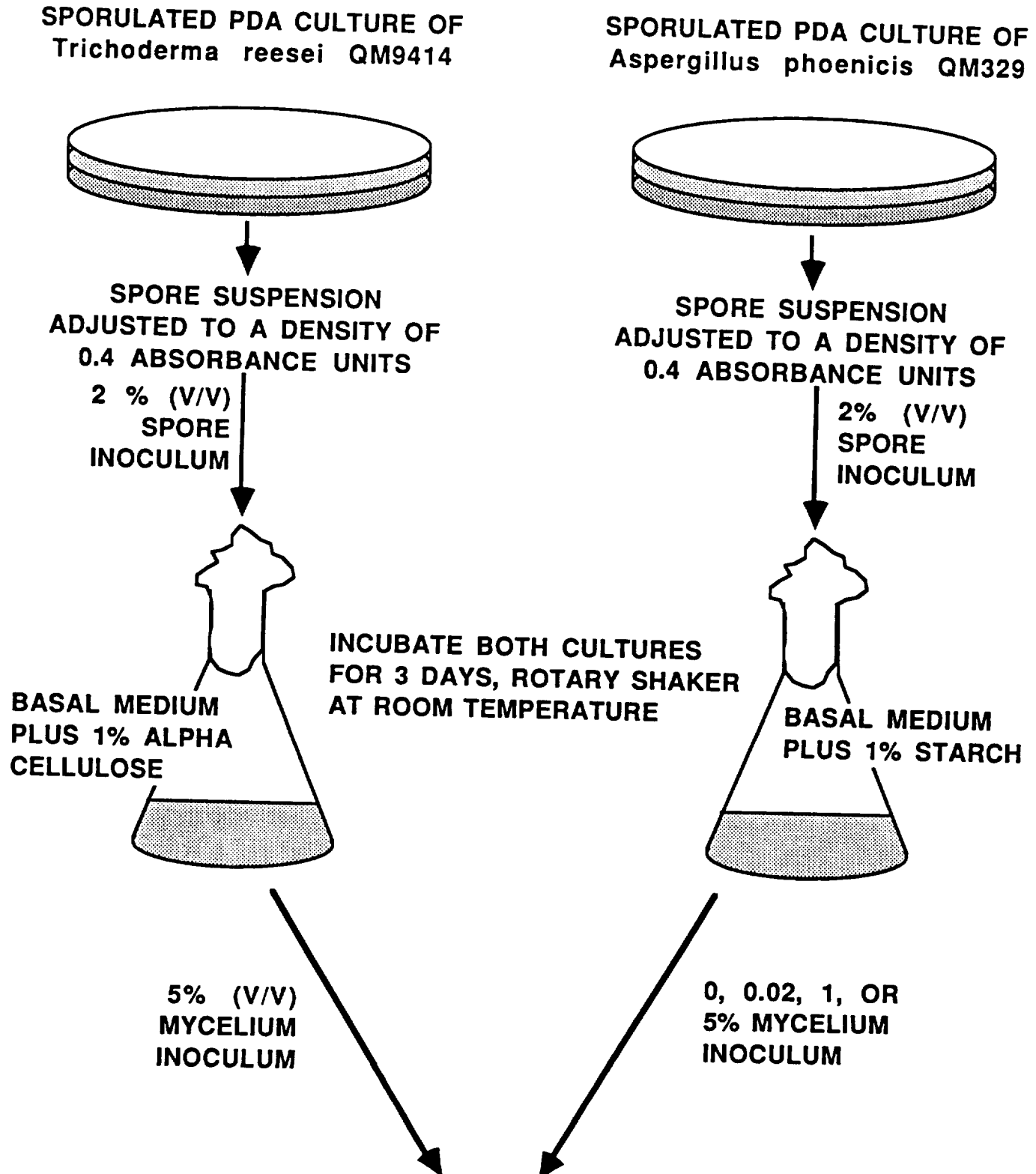
BACKGROUND

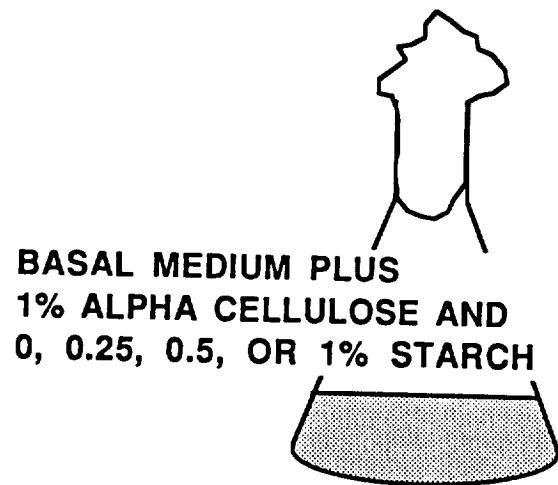
- 1) Cellulase is a complex composed of three major enzyme types: Endoglucanases, exoglucanases, and β -glucosidase.
- 2) *Trichoderma reesei* produces cellulase that is relatively deficient in β -glucosidase, which can result in the build-up of inhibitory levels of cellobiose.
- 3) Supplemental β -glucosidase can be produced by *Aspergillus phoenicis*, grown either separately or in mixed culture with *T. reesei*.
- 4) However, an additional carbon source, starch, is needed to grow the *A. phoenicis*.
- 5) Also, mixed cultivation may involve problems resulting from competition between the two fungi during growth.

PURPOSE

To evaluate the effects of inoculum density and starch concentration on the hydrolytic capacity of the cellulase complex produced in mixed cultures of *T. reesei* and *A. phoenicis*

EXPERIMENTAL APPROACH





**INCUBATE 7 DAYS ON A
ROTARY SHAKER FOR
PRODUCTION OF CELLULASE**

**SOLUBLE CELLULASE
ENZYME COMPLEX
HARVESTED**

ENZYME ACTIVITY ASSAYS

**B-GLUCOSIDASE
FILTER PAPER--REDUCING SUGAR
FILTER PAPER--GLUCOSE
SOLUBLE PROTEIN**

**NATIVE CELLULOSE
HYDROLYSIS ASSAY**

**24 AND 48 HR
GLUCOSE PRODUCTION
FROM WHEAT STRAW**

(BASAL MEDIUM: MINERAL SALTS, PEPTONE, AND TWEEN 80)

EXPERIMENTAL DESIGN

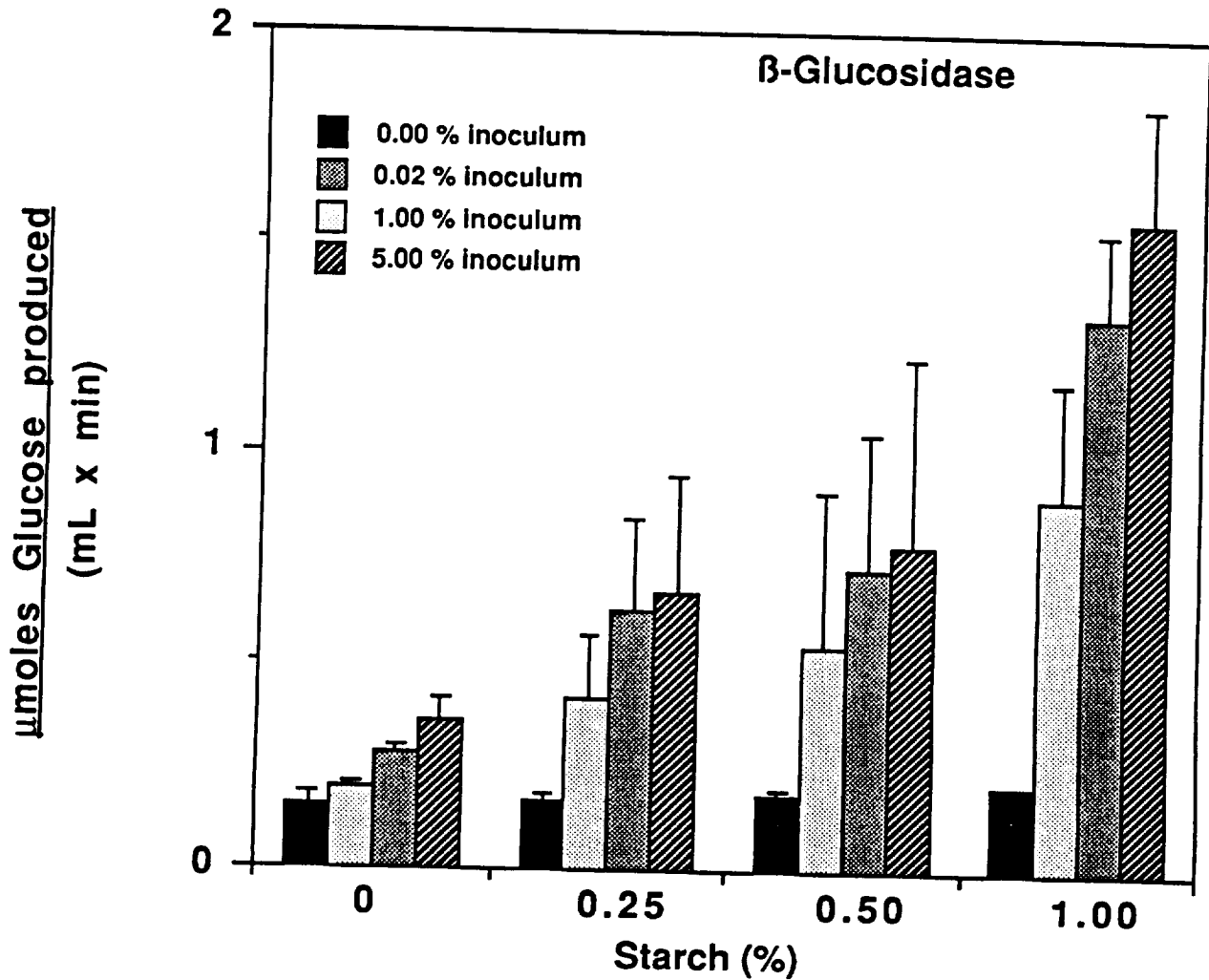
A 4 x 4 FACTORIAL DESIGN WAS UTILIZED

FACTOR	LEVEL
<i>A. phoenicis</i>	0, 0.02, 1, and 5%
Starch conc.	0, 0.25, 0.5, and 1%

Alpha cellulose concentration (1%) and *T. reesei* inoculum (5%) were held constant. Each treatment was run in triplicate.

ENZYME ACTIVITIES

β -Glucosidase Activity



- *A. phoenicis* inoculum density and starch concentration had a significant effect.
- The highest starch concentration (1%) caused significantly higher activities.
- Cultures with no *A. phoenicis* inoculum had significantly lower activities.

Follow-up studies

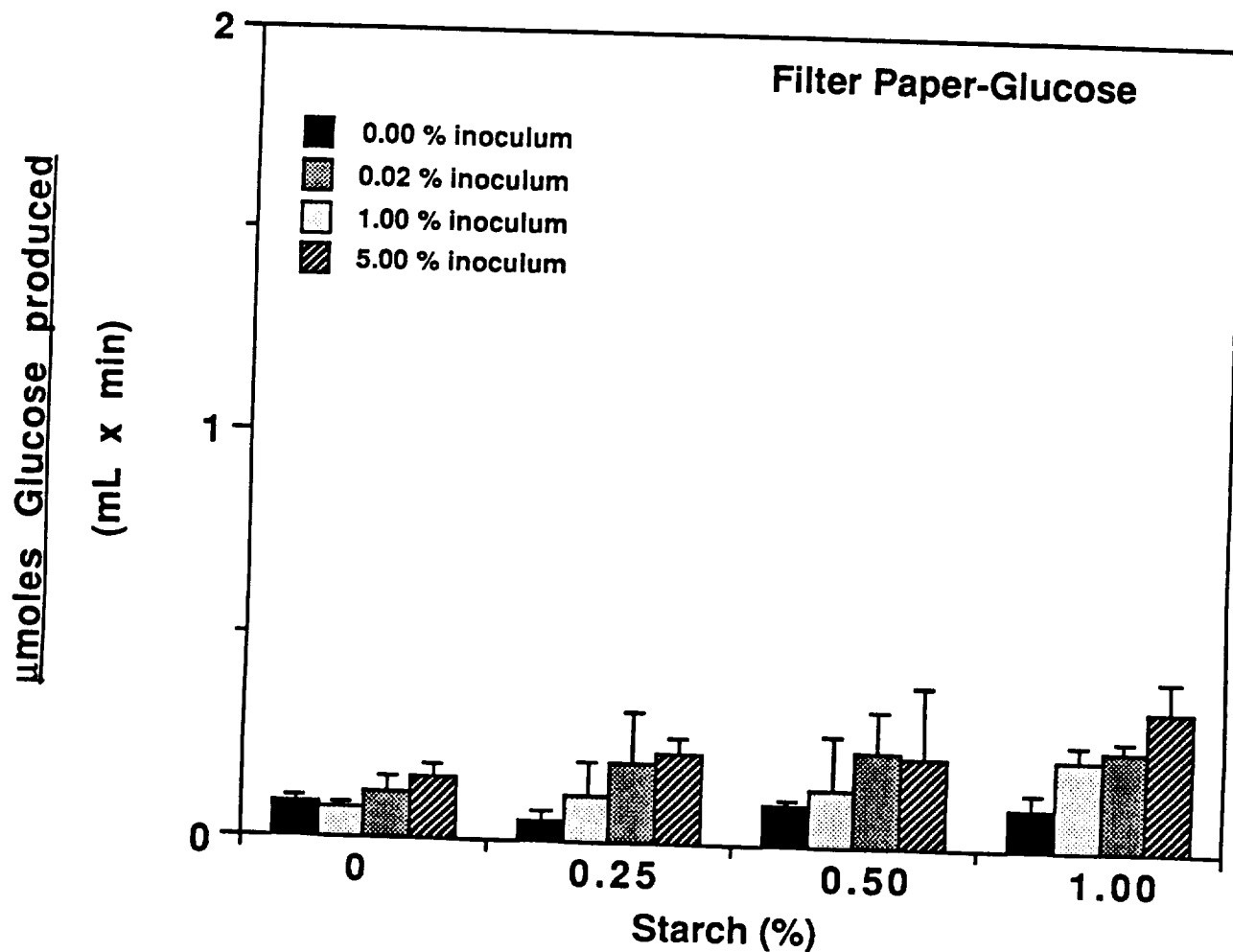
- Were performed to determine if increased β -glucosidase activity was a result of introduction of starch or enzyme with the *A. phoenicis* inoculum.
 - Washing the *A. phoenicis* inoculum prior to introduction into the co-cultures had no effect on the subsequent β -glucosidase activity
 - Cultures that received 2.5% inoculum of both *A. phoenicis* and *T. reesei* did not have significantly less β -glucosidase activity than cultures that received 5% inoculum of both fungi.

Enzyme Activity--Correlations

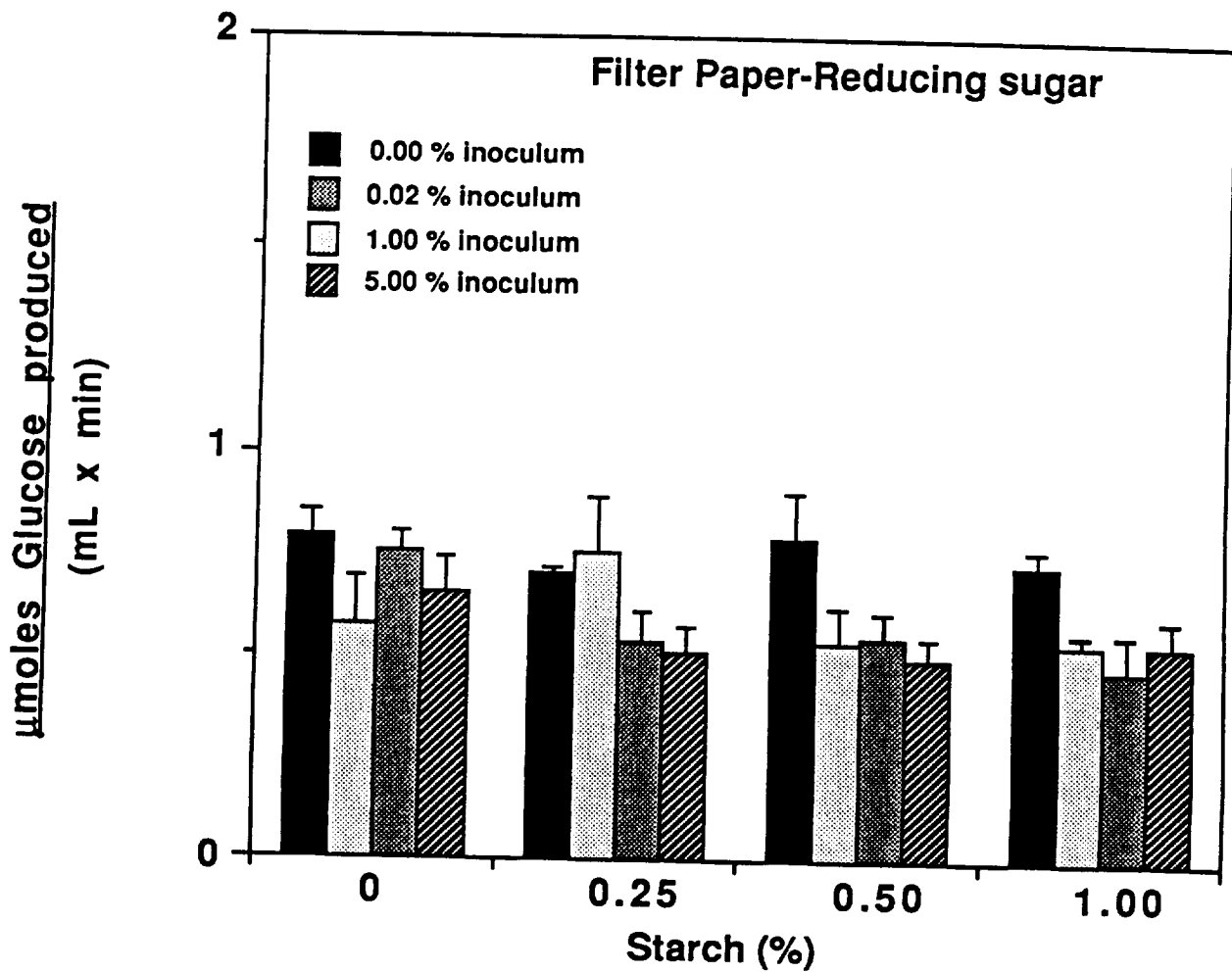
- Between Filter Paper--glucose activity and β -glucosidase activity is strong ($r=0.83$). Rapid hydrolysis of oligosaccharide intermediates (particularly cellobiose) at higher β -glucosidase activities may be the cause.
- Between Filter paper--reducing sugar and β -glucosidase activity is weak and negative ($r= - 0.56$). *A. phoenicis* growth may have an inhibitory effect on the production of endo- and exo-glucanases by *T. reesei*. Further study is necessary to conclusively prove any causation.

Filter paper activity

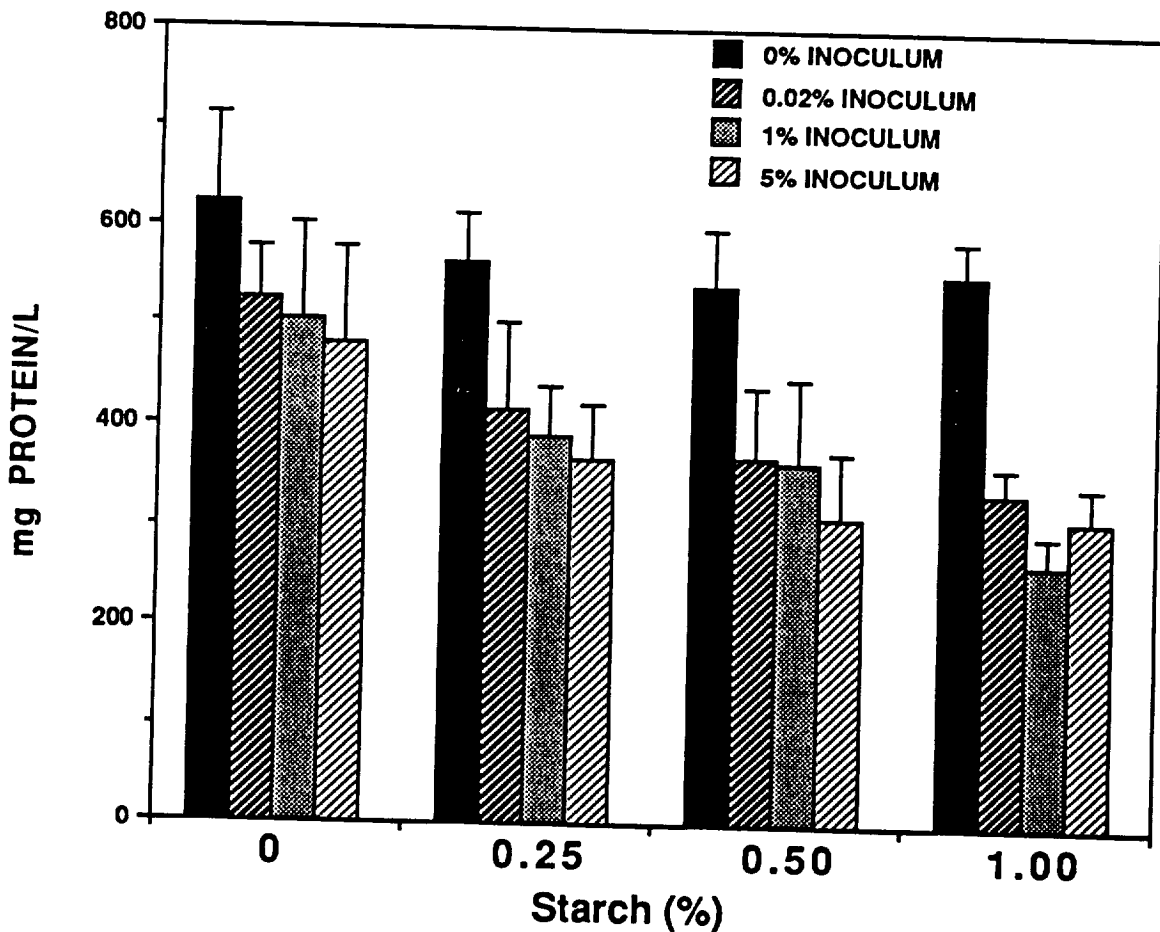
- Based on glucose production--significantly increased at higher levels of both factors.



- Based on production of reducing sugars (below)--decreased in co-cultures which contained starch. The DNSA method overestimates the concentration of glucose because it also measures oligosaccharides.



SOLUBLE PROTEIN



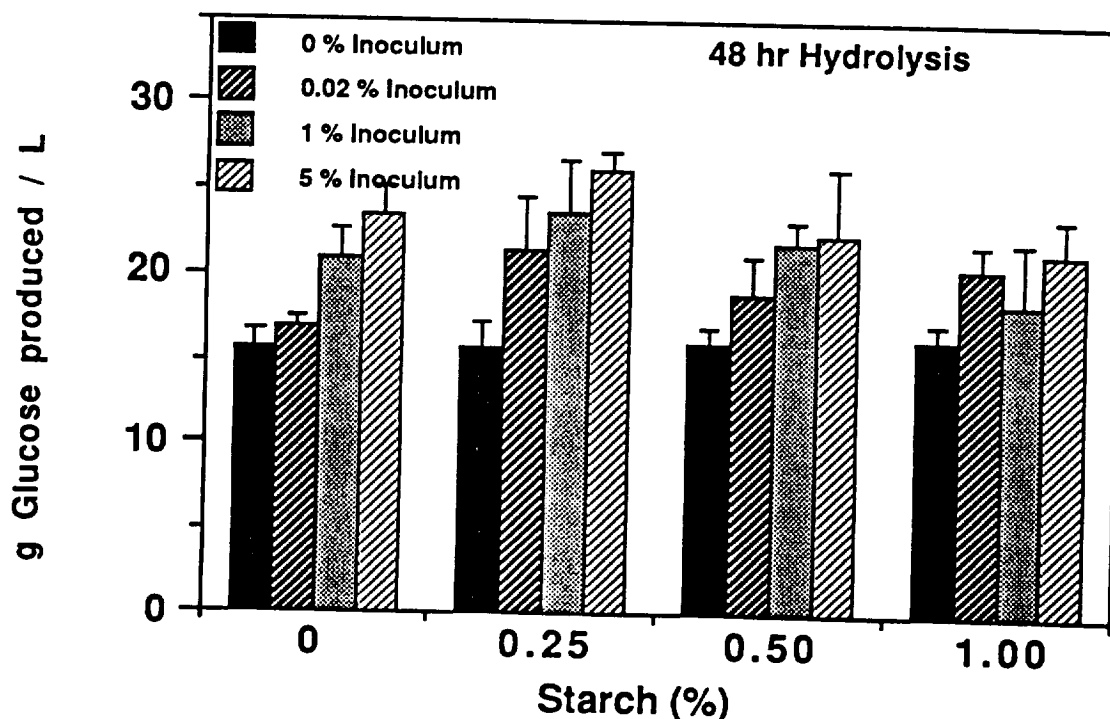
- In co-cultures, soluble protein decreased significantly with both increasing *A. phoenicis* inoculum and starch concentration.

- * In 7 day old monocultures, *A. phoenicis* produced 10 μg soluble protein per mL compared to 700 μg per mL by *T. reesei*.

- Thus, lower soluble protein levels in co-culture may be a result of increased *A. phoenicis* biomass at the expense of *T. reesei*.

- A significant competitive interaction between the two fungi appears to occur when starch is present.

WHEAT STRAW SACCHARIFICATION

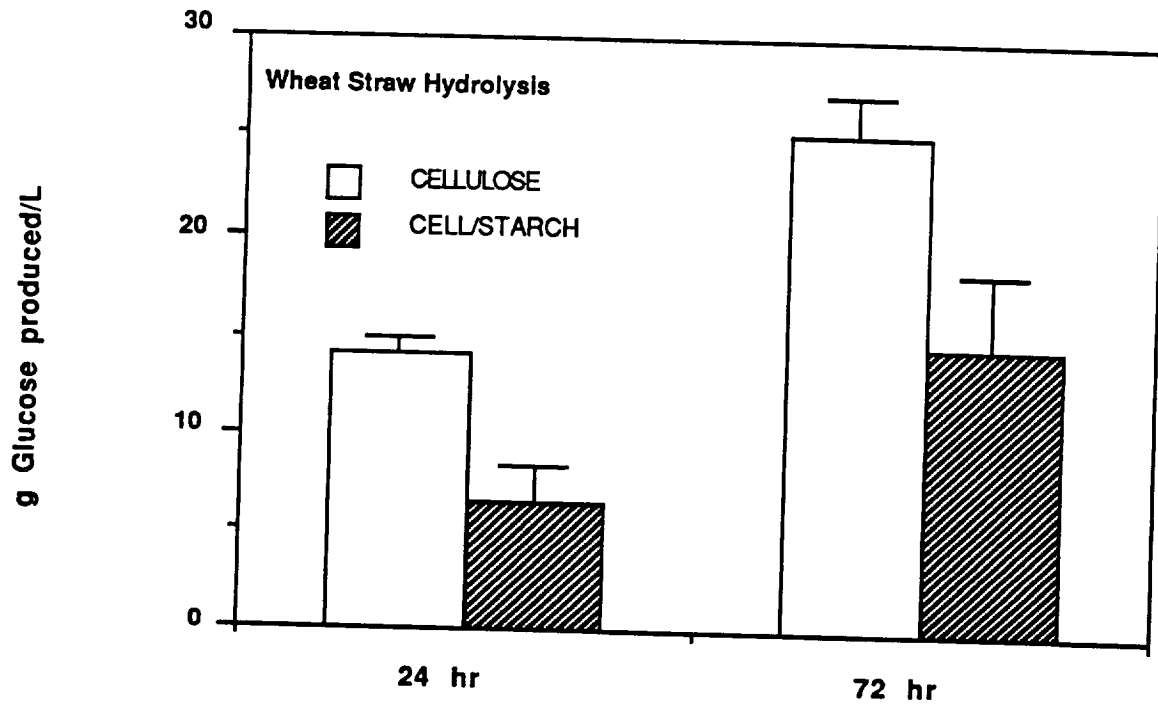


- *A. phoenicis* inoculum density had a significant positive effect on glucose production from wheat straw.
- Starch concentration in co-cultures did not have a significant effect but caused a slight depression in glucose production at higher levels

Correlations

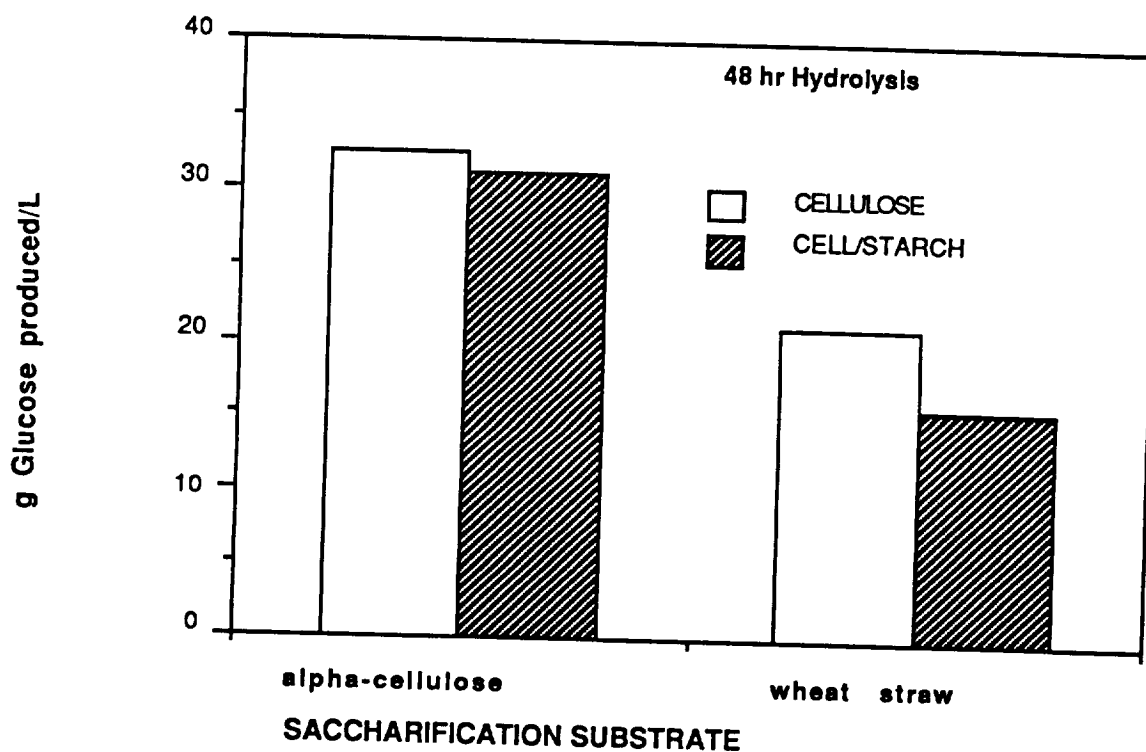
- Between glucose production from wheat straw and the enzyme assays are low.
- This reveals the limitations of depending on enzyme assays alone to predict the hydrolytic potential of a cellulase complex acting on a natural cellulosic substrate.

FOLLOW-UP STUDIES



Follow-up studies were performed to verify the decrease in hydrolytic potential in co-cultures containing higher levels of starch.

- Glucose production from wheat straw hydrolysis was significantly greater after 24 and 72 hours using enzyme from co-cultures containing only 1% cellulose compared to enzyme from co-cultures containing both 1% cellulose and 1% starch.



- Glucose production from alpha-cellulose after 48 hours of hydrolysis was the same for enzymes harvested from both of the above co-cultures.
- This result indicates that the recalcitrance of native cellulose, i.e., wheat straw, may be an important factor in determining the relative hydrolytic capacity of cellulase produced in co-culture.

CONCLUSIONS

- 1) Cellulase production in co-cultures of *Trichoderma reesei* and *Aspergillus phoenicis* is significantly affected by starch concentration and relative inoculum density.
- 2) Co-cultures without starch produced a cellulase with increased β -glucosidase activity when compared to monocultures of *Trichoderma reesei* and with greater hydrolytic potential toward a natural cellulosic substrate when compared to co-cultures that contained starch.
 - *A. phoenicis* β -glucosidase may be induced by oligosaccharides produced during the hydrolysis of cellulose by the *T. reesei* cellulase complex.
- 3) Co-cultivation with cellulose alone eliminates the added cost of separate enzyme production and recovery processes needed to produce supplemental β -glucosidase without the use of additional carbon sources.